Original Article

Anti-*Acanthamoeba* Activities of Chloroformic Fractions of *Trigonella Foenum Graecum* (Seed) and Their Cytotoxity on Mice Macrophage Cell

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Abstract

Background: *Acanthamoeba keratitis* (AK) is potentially a sight-threatening infection and its treatment is challenging. This is mainly due to presence of resistant cyst form. Indeed, cysts are highly resistant to current available drugs. Chemical drugs are toxic to human keratocytes. It should also be mentioned that most available anti-*Acanthamoeba* drugs are poorly cysticidal, In Iran and worldwide, AK cases continue to rise and therefore, novel effective drugs are urgently needed for the treatment of AK.

Materials and Methods: In the present study, the *in vitro* activity of serial dilutions (10, 15, 20 and 25 mg/mL) of chloroformic fractions including primary chloroformic fraction (minimum amount of chloroform), middle chloroformic fraction and remaining chloroformic fraction (most amount of chloroform) of *Trigonella foenum graecum* seed were evaluated against *Acanthamoeba* trophozoites and cysts. Cytotoxic assay of fractions at different concentrations (25, 50, 100, 200, 300, 400, 500 mg/ml) of test material was identified on mice Macrophage cells using MTT method.

Results: The obtained results revealed that the tested fractions presented anti-amoebic activities in a time and dose dependent cycle. Anti-*Acanthamoeba* activity of remaining chloroformic fraction was more than other fractions. Trophozoites/cysts were eliminated when incubated with 15 and 20 mg/ml concentrations of remaining chloroformic fraction after 24 hours. Viability of macrophage cells was noted 100 % with 25 and 50 mg/ml concentration of remaining chloroformic fraction. Our results indicate that the plant fractions are safe for mammalian cells.

Conclusion: Further studies should be performed in order to detect the active chemical compounds which could be used for the development of novel therapeutic approaches against *Acanthamoeba* infections. To the best of our knowledge, this is the first study on the activity of chloroformic fractions of *Trigonella foenum graecum* (seed) against *Acanthamoeba* spp.

Keywords: *Acanthamoeba*, Chloroformic fractions, *in vitro*, Therapy

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Introduction

Acanthamoeba spp. is a genus of free-living protozoa. This protozoa is introduced as a widespread organism, and can be found on every continent, as well as in air-conditioning units, in water mains,

showers, sanitary and dental equipment, contact lens care solutions, and infected tissue cultures. The life cycle of *Acanthamoeba* consists of a motile trophozoite and a double-walled cyst[1].

Invasion in the human can cause fatal encephalitis in the immunocompromised patients[2] and potentially sight-threatening ocular infection termed *Acanthamoeba* keratitis[3]. Amoebic keratitis is a potentially severe ocular infection characterized by progressive corneal ulceration. If *Acanthamoeba* keratitis is not diagnosed and treated early, it could be lead to perforation, ring infiltration of corneal tissue and finally loss of vision[1,4]. Eradication of these amoebas is difficult because, under inappropriate conditions, the amoebas are encysted and the current therapy is often less effective against cyst form than trophozoite form[5]. *Acanthamoeba* keratitis is usually treated with a combination of cationic antiseptics such as polyhexamethylene biguanide and aromatic diamidines such as propamidine isethionate[6,7]. Chlorhexidine, alone or in combination with propamidine isethionate[8,9] is another form of treatment options. The current drugs against the *Acanthamoeba* exhibit many toxic effects on corneal cells[10,11]. This leads to severe interest in search of safer drug for the treatment. *Trigonella foenum-graecum* L. (Fungreek) is an erect, scented annual herb that is extensively cultivated in warm temperate and tropical regions in India, the Mediterranean region, North Africa, and Iran[12]. Seeds has been documented for its different pharmacological activities including hypoglycemic[13,14] gastro-protective and anti-ulcer effects[15]. The leaves were also evaluated for potent antinociceptive, anti-inflammatory and antipyretic activities[16]. Researches revealed that the major components of the plant seed are saponins, glycoside-D and trigofoenoside-A[17], while in the leaves are found alkaloids, cardiac glycosides, and phenols[16].

The present study was planned to investigate amoebicidal activity of the partially separated fraction obtained from the *Trigonella foenum-graecum* seed extract on the growth of *A. castellanii* trophozoites and cysts and cytotoxic activities of fractions on mice Macrophage cell.

Methods

Preparation of chloroformic fractions of the plant seed

The dried seeds of the plant were purchased from the Ray area of Iran and carefully identified and approved by pharmacognosist (Pharmacognosy school, Shahid Beheshti University of Medical Science, Tehran, Iran). Dried coarsely powdered seeds (500 g) were extracted with boiling distilled water using Soxhlet apparatus at 80±2°C for 10 hours. The solvent was evaporated by distillation and under vacuum. To obtain the fractions successive liquid-liquid partitioning was used. The raw sticky extract was subsequently adjacent to chloroform several times. Thus three separate phase including primary chloroformic fraction (minimum amount of chloroform), middle chloroformic fraction and remaining chloroformic fraction (most amount of chloroform) were obtained[17].

***Acanthamoeba* strain**

The *Acanthamoeba* strain isolated in this study was obtained from a patient with AK. Diagnosis of AK was based on culture and sequencing based tests. The specimen was inoculated onto the surface of 1.5% non-nutrient agar (NNA) along with *Escherichia coli* and incubated at 26˚C for 72 hours. PCR was used to confirm the microscopic identification. Sequencing followed by BLAST search of the isolate revealed the presence of T_4 genotypes. Cloning of the amoeba was done in order to eliminate any bacterial and fungal contamination of the plates[18,19]. The *Acanthamoeba* strain was then kept in NNA culture until further use.

Trophozoites preparation

Acanthamoeba Spp. was grown in axenic medium according to our previous study[20]. Trophozoites in the stage of exponential growth (72 to 96 h) were identified by inverted microscope. For the experiment, one ml of the culture was centrifuged (5 min at 2000g) and the sediment washed twice with phosphate-buffered saline buffer (PBS). The trophozoites viability was determined using eosin 0/1% and direct trophozoite counts were done using a neubauer cell-counter chamber. Final concentration was adjusted to 25×10^4 trophozoites per ml for the activity assays[21,22].

Cysts preparation

Axenic medium was inoculated directly onto the surface of 1.5% non-nutrient agar (NNA) plates and

incubated in a humidified chamber at 26˚C. Cysts were identified by microscope after several days. The agar surface was then flooded with 5 ml of PBS and were scraped with a sterile loop. Cysts were harvested from the suspension by centrifugation (1500 g for 5 min). The sediment was then washed twice in PBS. Cysts in the obtained suspension were counted with a neubauer cell-counter chamber, and the suspension was adjusted to 25×10^4 cysts per milliliter for the cystidical activity assays[22,23].

Evaluation of activity

Plant chloroformic fractions were prepared according to following concentrations: 10, 15, 20 and 25 mg/mL. To this end, 100-150 µl of the calibrated cyst/ trophozoite suspension (25×10^4/ml) was inoculated in each microcentrifuge tubes and 150µl of each extract concentration was added into the tubes. Incubation was done at 26˚C with different incubation periods (24, 48 and 72 h). In addition, untreated control (negative control) containing only the parasite plus PBS (without extracts) and treated control (positive control) containing the parasite plus 0.02% chlorhexidine gluconate (prepared from a solution 20% in water CHX, C-9394; Sigma) were used. Three tubes were used for the evaluation of each concentration and measurements were repeated 5 times[24].

Fractions effect against trophozoite and cyst stage

Twenty five µl from each test and control well was added into the same volume of 0.1% eosin. The number of amoebae were identified by means of an inverted microscope and trophozoites and cysts were counted in a counting chamber after each incubation period. Viability was assessed using eosin 0/1% and approximately 100 *Acanthamoeba* trophozoites were examined in each time. Additionally, the cultures containing no viable cysts were transferred onto a NNA agar coated with *E. coli* and incubated at 26˚C during another three days to confirm the observed results[24].

Cytotoxic assay of fractions on Mice Macrophage cells using MTT test

Mice Macrophage cells were obtained from peritoneal cavity of mice for the experiment followed by culture in RPMI-1640 medium. The other step was the removal of 75µl (15000 cell) from the suspension and the cells were transferred to wells of 96-well plate. The cells were then kept at 37° C in a 5% CO2 air incubator and passaged every day. Ten µl of fractions with different concentrations (25-500 mg/ml) were added separately to all wells except the controls and the plates were mixed well. The cells were maintained at 37°C in a 5% CO2 air incubator along 24 hours. After incubation period, cells viability were determined using MTT assay. Briefly, cells grown in a 96-well plate, were incubated with the MTT solution approximately 4 hours. Then a formazan dye was quantitated using a ELISA reader 540 nm wavelength after 15 minutes (viability (%)=O.D. TESE/O.D. CONTROL X 100)[25].

Statistical analysis

The statistical analyses of data were performed using SPSS software version 15.0. P values<0.001 were considered as statistically highly significant. The IC50 analysis was done using GraphPad Prism 6.

Results

The present study was detected the amoebicidal activity of the chloroformic fractions of the seeds of *Trigonella foenum graecum*. Interestingly, remaining chloroformic fraction had a high amoebicidal activity (MIC=15, 20 mg/ml) against trophozoites and cysts, however primary chloroformic fraction and middle

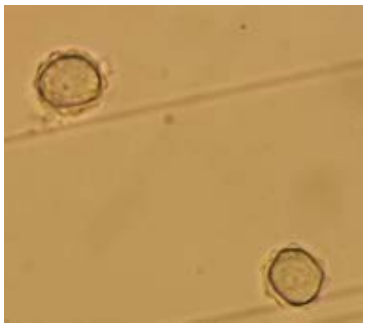

Figure 1. Cyst before treatment of chloroformic fractions (x400)

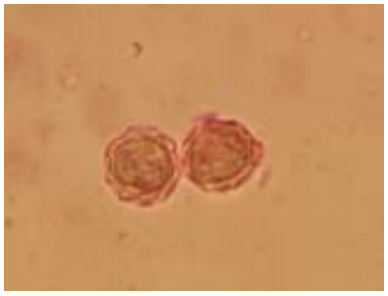

Figure 2. Cysts after treatment of chloroformic fractions (x400)

Table 1: Effect of *Trigonella foenum graecum* chloroformic fractions on the survival and growth of *Acanthamoeba trophozoite* and cysts.

Compounds	concentrations(mg/ml)	Amoebae form	Live parasites in	24h	48h	72h
MTH_1	10	Trophozoites		9/73±0/83	1/8±0/86	0±0
		Cysts		12/27±1/66	2/8±1/52	0±0
	15	Trophozoites		7/93±1/38	0±0	0±0
		Cysts		8/33±1/04	0±0	0±0
	20	Trophozoites		3/20±1/08	0±0	0±0
		Cysts		5/53±1/12	0±0	0±0
	25	Trophozoites		0±0	0±0	0±0
		Cysts		0±0	0±0	0±0
MTH_2	10	Trophozoites		6/0±1/64	1/0±1/25	0±0
		Cysts		9/0±1/81	2/5±1/87	0±0
	15	Trophozoites		4/0±1/41	0±0	0±0
		Cysts		7/07±1/66	1/9±1/66	0±0
	20	Trophozoites		1/8±1/1	0±0	0±0
		Cysts		3/2±1/4	0±0	0±0
	25	Trophozoites		0±0	0±0	0±0
		Cysts		0±0	0±0	0±0
MTH_3	10	Trophozoites		5/2±1/43	0±0	0±0
		Cysts		8/5±1/2	0/5±0/1	0±0
	15	Trophozoites		0±0	0±0	0±0
		Cysts		3/2±1/2	0±0	0±0
	20	Trophozoites		0±0	0±0	0±0
		Cysts		0±0	0±0	0±0
	25	Trophozoites		0±0	0±0	0±0
		Cysts		0±0	0±0	0±0

MTH_1: primary chloroformic fraction
MTH_2: middle chloroformic fraction
MTH_3: remaining chloroformic fraction

Table 2: Effect of chloroformic fractions of *Trigonella foenum graecum* on % viability of mice Macrophages after 24h.

Concentrations (mg/ml)	%Viability of cells after effect of MTH_1	%Viability of cells after effect of MTH_2	%Viability of cells after effect of MTH_3
25	73.7%	84.8%	100%
50	65.9%	76.2%	100%
100	57.7%	74.0%	90.0%
200	52.6%	64.1%	88.6%
300	50.6%	56.8%	75.0%
400	49.9%	54.8%	71.6%
500	26.2%	32.9%	56.5%

MTH_1: primary chloroformic fraction
MTH_2: middle chloroformic fraction
MTH_3: remaining chloroformic fraction

chloroformic fraction had less activity against amoebae than remaining chloroformic fraction (Table

1). On the other hand, at 20 mg/ml concentration of primary chloroformic fraction 3.2×10^4 and 5.53×10^4 trophozoites and cysts were lived after 24 hours. In same concentration of middle chloroformic fraction 1.8×10^4 and 3.2×10^4 trophozoites and cysts were lived. Light microscopy revealed that the untreated *Acanthamoeba* cyst contained double walls: the intact ectocyst and endocyst walls. The endocyst appeared smooth and a clear space separated it from wrinkled ectocyst and non-treated trophozoite showed acanthopodia. Cysts treated with the extract showed a cytoplasmic clump and rounded walls and even some cysts presented empty walls (Figures 1, 2). Nevertheless, it is important to mention that a time and dose dependent amoebicidal action of the extract was shown on both stages of the amoebae. The 24-hour cytotoxic effects of chloroformic fractions of *Trigonella foenum graceum* (TFG) was surveyed on macrophage cells using the MTT test. A dose dependent MTT reduction was observed in TFG exposed cells. Fractions were added in different concentration from 25 to 500mg/ml to the macrophage cells and incubated for 24 hours. Viability was noted 100% for the 25 and 50 mg/ml concentration of remaining chloroformic fraction (IC_{50}= 639.6mg/ml) although it estimated 73.7% (IC_{50}=215.4mg/ml) and 84.8% (IC_{50}=348.6mg/ml) in 25mg/ml concentration of primary chloroformic fraction and middle chloroformic fraction respectively (Table 2).

Discussion

*Acanthamoeb*a keratitis is a severe corneal disease related to the users of soft contact lenses. This sight threatening corneal disease is often treated with combination drugs such as polyhexamethylene biguanide (PHMB) or chlorhexidine and propamidine isethionate. Despite combination therapy, only half of the patients have been reported to improve after treatment with the therapeutic regimens when the disease is not early diagnosed[26]. According to previous researches, biguanides could lead to toxic effects on human keratocytes even at the lowest cysticidal concentrations[27]. Long duration of treatment course for the mentioned drugs is notable and it may even take up to six months[28]. Poor cysticidal effect is the main drawback to the mentioned drugs [29,30]. Thus, cysts resistance to current available drugs have prompted the researchers to develop novel anti-amoebic compounds. To date, there is a raising pattern to shift resources from currently chemical drugs to herbal medicine. Indeed, finding a natural compound with amoebicidal and cysticidal effect and non-toxic to human cells is of utmost priority to overcome AK cases.

In our study, the activity of the chloroformic fractions of *Trigonella foenum graecum* seed was evaluated against a pathogenic *Acanthamoeba* strain since a previous study reported that this medicinal plant presented anti-parasitic properties when assayed in various parasites[32,33]. The present study showed that the tested chloroformic fractions were able to eliminate trophozoites and cysts of *Acanthamoeba*. However, we tested a higher concentrations (500 mg/ml) in comparison with other medicinal plants and thus cytotoxic evaluation on macrophage cells was carried out. The results confirmed remaining chloroformic fraction has no cytotoxic effect on the macrophage cells with the dose of 50 mg/ml.

In addition, *T. foenum graecum* is a widely used medicinal plant in various disorders. Indeed, according to previous researches antidiabetic, anti-inflammatory, antipyretic[34], anthelmintic and antibacterial properties were elucidated[35]. Interestingly, previous studies revealed that oral administration of *Trigonella foenum graecum* can cause a significant protection against eye cataract in animal model[36].

Conclusion

Overall, the present research demonstrated that remaining chloroformic fraction of *T. foenum graecum* could be used in the near future in order to obtain amoebicidal and cysticidal compounds for the treatment of *Acanthamoeba* infections. This is mentionable that these fractions have maximum effect on *Acanthamoeba* during 24 hours, thus there is no need for more exposure time. Toxicity tests reflected that the mentioned fraction have none side-effects. Further *in vitro* and *in vivo* studies are necessary to perform in order to introduce the chemical molecular component of remaining chloroformic fraction involved in the anti-*Acanthamoeba* activities.

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